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## Introduction

A hallmark of metastatic cancer is the dissemination of tumor cells via the blood stream or the draining lymphatic system. Whereas normal epithelial cells and non-invasive cancers are typically separated from blood vessels by a basement membrane, invasive tumors destroy this barrier and experience a dramatic change in their extracellular environment. One aspect is the exposure of the tumor cells to components of the blood, either due to the extravasation of blood plasma proteins from leaky tumor-associated vessels or due to the entry of the cells into the blood stream. It has long been recognized that tumor cells express potent procoagulants, such as the cell surface receptor tissue factor (TF) that triggers activation of the plasma coagulation system<sup>1</sup>. Upon exposure to the blood, tumor cells activate the highly ordered cascade of coagulation factors on the tumor cell surface, resulting in thrombin generation. This enzyme activates the G-protein coupled thrombin-receptor by a unique proteolytic mechanism<sup>2</sup>. Important recent evidence demonstrates that the thrombin receptor is not detectable in non-invasive ductal carcinoma in situ, but is highly upregulated in invasive breast cancer<sup>3</sup>. Thus, invasive tumor cells are the target for the activating effects of thrombin, as soon as the cells are exposed to plasma components or enter the blood stream.

The goal of this proposal was to identify genes that are upregulated upon thrombin stimulation. Thrombin mediated expression of certain genes was hypothesized to change the tumor microenvironment and the invasive or metastatic potential of breast cancer cells. The identified genes may represent novel targets for therapy of invasive and metastatic breast cancer. The approach was to use one prototypical breast cancer cell line with a documented response to thrombin. Thrombin cleaves and activates protease activated receptor (PAR) 1, a member of the G-protein coupled receptor family. In these experiments, my laboratory performed a comprehensive gene profiling for thrombin and PAR-1 induced genes in breast cancer.

## Body

### **1. Characterization of PAR-1 expression and PAR-1-dependent activation of the MAP kinase pathway in breast cancer cells.**

Activation of PARs leads to phosphorylation of mitogen activated (MAP) kinase Erk1/2 in serum-starved cells. A total of 6 breast cancer cell lines were screened for Erk1/2 phosphorylation in response to activation by thrombin. Two estrogen receptor positive cell lines (HCC70, HCC1500) showed no thrombin response in this assay. Among the estrogen-receptor negative cell lines, cells with an epithelial morphology showed no signs of thrombin response, while more dedifferentiated cell lines had responses to thrombin or showed constitutive upregulation of the MAP kinase pathway. Typical examples for the responses to thrombin stimulation and to stimulation with specific agonists that activate PAR-1, 2, or 4 is shown in Fig. 1. The cell lines fell in three distinct categories. (1) Cells had upregulated Erk1/2 phosphorylation after serum starvation and showed little (e.g. MDA-MB231, not shown) or non-detectable (MDA-MB435) upregulation of Erk1/2 phosphorylation. (2) Certain cells with low levels of Erk1/2 phosphorylation responded only to stimulation with a PAR-2 agonist (BT-20). PAR-2 is known

to be present in primary epithelial cells <sup>4</sup>. (3) Other cells with low constitutive Erk1/2 phosphorylation responded to both PAR-2 and PAR-1 agonists and thus acquire thrombin responsiveness that can be blocked with antibodies which prevent the cleavage and activation of PAR-1 (743B, Hs578T). Expression of PAR-1 was confirmed on these cell lines by flow cytometry (Table 1). Notably, the highly metastatic MDA-MB435 had the highest levels of PAR-1 expression of all cell lines. However, the constitutive upregulation of the MAP kinase excluded a fast confirmation of a successful stimulation for the profiling experiment. The decision was made to proceed with Hs578T that showed a highly reproducible PAR response.

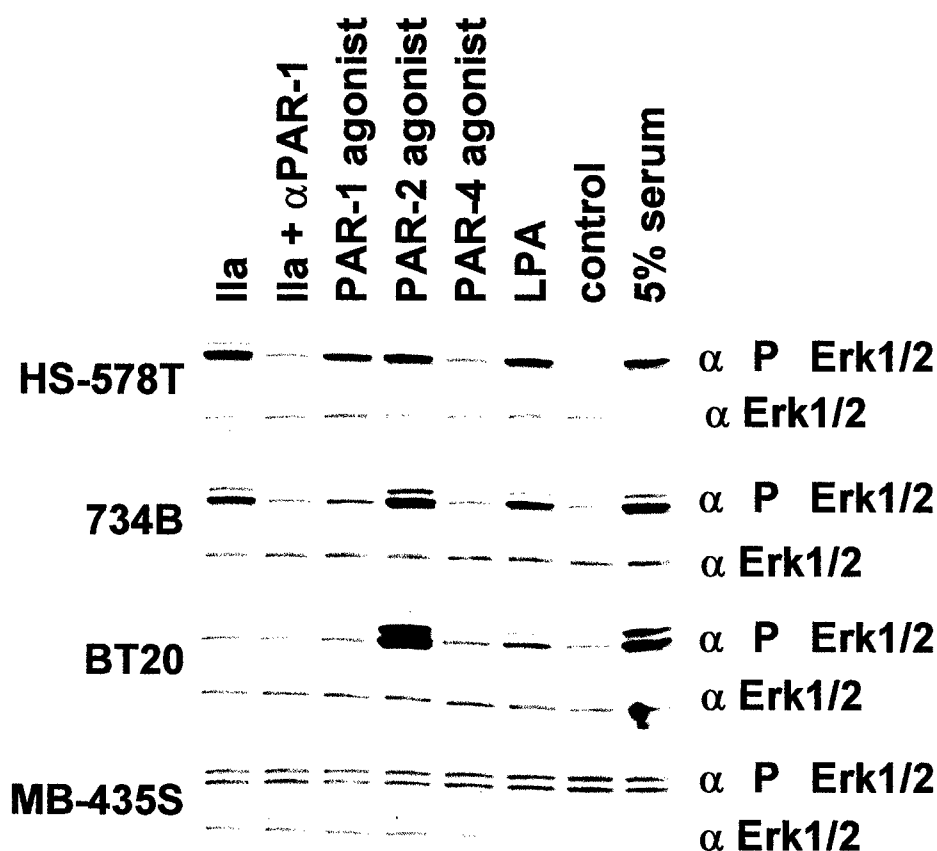


Fig. 1:  
Characterization of thrombin responses in breast cancer cell lines. Serum-starved cells were stimulated with 10 nM thrombin (IIa), 10  $\mu$ M TFLLRNPNDK (PAR-1 agonist), 100  $\mu$ M SLIGRL (PAR-2 agonist), 500  $\mu$ M AYPGKF (PAR-4 agonist). Stimulation with LPA, that activates unrelated G-protein coupled receptors was used as a control along with serum stimulation.

Table 1: Expression of PAR-1 by Breast Cancer Cells

	Mean Fluorescence		
	$\alpha$ PAR-1 (ATAP2)	$\alpha$ PAR-1 (WEDE15)	$\alpha$ TF
Control (HUVEC)	55	52	0
Hs578T	31	24	160
743B	9	6	45
BT-20	2	0	41
MDA-MB435s	124	140	24

## 2. Validate Affymetrix gene chip technology and determine optimal time of stimulation.

The relatively inexpensive cancer chip array (representing approximately 1200 genes of relevance for cancer) was used for the initial experiments to validate the technology and define optimal stimulation times. Cells were stimulated for 1, 3, and 16 hours and each sample was hybridized to two separate arrays. Fig. 2 shows the correlation of the duplicate hybridizations.

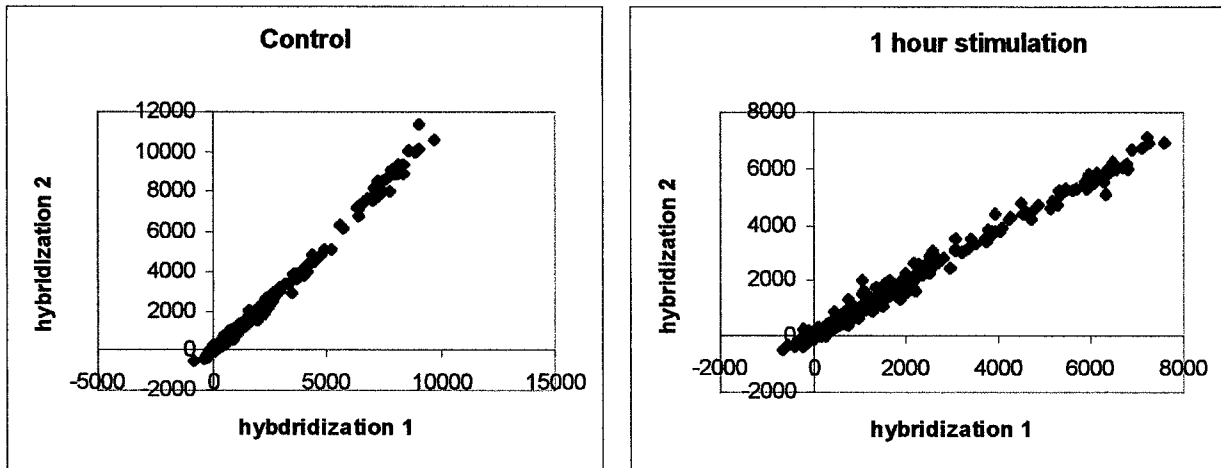


Fig. 2: Reproducibility of the hybridization to Affymetrix Human Cancer Gene Chip.

**Conclusion:** The methodology is highly reproducible and hybridization to a single chip is adequate.

## 3. Characterize cancer related genes that are upregulated by thrombin stimulation.

There was good correlation between the induction of genes at 1 and 3 hours, whereas several genes returned to baseline levels at 16 hours. An independent experiment with a 3 hour stimulation was performed to provide independent confirmation of the gene induction event. Table 2 shows the average for the 3 hour time point of the most highly induced genes in Hs578T breast cancer cells that were stimulated with thrombin (10 nM). These genes were also found to be upregulated after one hour of stimulation.

Table 2: Genes expressed at 3 hours on the Human Cancer Chip.

Gene	Fold induction 3 hours
M28130mRNA Human interleukin 8 (IL8) gene, complete cds	38.6
D30037 Human mRNA for phosphatidylinositol transfer protein (PI-TPbeta), complete cds	9.2
U15932 Human dual-specificity protein phosphatase mRNA, complete cds	6.7
Y00083cds Human mRNA for glioblastoma-derived T-cell suppressor factor G-TsF (transforming growth factor-beta2, TGF-beta2)	5.7



U12471cds#2 thrombospondin-p50 gene extracted from Human thrombospondin-1 gene, partial cds	4.2
X68277cds Homo sapiens CL 100 mRNA for protein tyrosine phosphatase	4.2
M26683 Human interferon gamma treatment inducible mRNA	4.0
M29039cds HUMJUNCAA Human transactivator (jun-B) gene; complete cds.	3.6
J03764cds HUMPAIA Human; plasminogen activator inhibitor-1 gene; exons 2 to 9.	3.6
M59465 Human tumor necrosis factor alpha inducible protein A20 mRNA, complete cds	3.3
M65254 Protein phosphatase 2A 65 kDa regulatory subunit-beta mRNA, complete cds	3.2
Z36714mRNA Homo sapiens mRNA for cyclin F	3.2
L26336cds Human heat shock protein HSPA2 gene, complete cds	3.1
U77949 Human Cdc6-related protein (HsCDC6) mRNA, complete cds	3.1
U45878 Human inhibitor of apoptosis protein 1 mRNA; complete cds.	3.1
S75881 A-myb=DNA-binding transactivator {3' region} [human; CCRF-CEM T-leukemia line; mRNA Partial; 831 nt]	3.0
L13740 HUMTR3A Human TR3 orphan receptor mRNA; complete cds.	3.0
U68019 Human mad protein homolog (hMAD-3) mRNA, complete cds	2.8
M58603 Human nuclear factor kappa-B DNA binding subunit (NF-kappa-B) mRNA, complete cds	2.8
M97935 Homo sapiens transcription factor ISGF-3 mRNA, complete cds (_5, _MA, MB, _3 represent transcript regions 5 prime, MiddleA, MiddleB, and 3 prime respectively)	2.7
U24153 Human p21-activated protein kinase (Pak2) gene, complete cds	2.7
U69108 Homo sapiens TNF receptor associated factor 5 mRNA, partial cds	2.7
M33684cds HUMPPP1A5 Human (clone lambda-16-1) non-receptor tyrosine phosphatase 1 (PTPN1) gene; exon x+4 and 5' end cds.	2.6
D38449 Human mRNA for G protein-coupled receptor, complete cds	2.6
M28212 Homo sapiens GTP-binding protein (RAB6) mRNA, complete cds	2.6

The gene induction profile indicates a complex transcriptional response that, in part, involves the activation of the MAP kinase pathway. In addition, induction of genes associated with the NF $\kappa$ B pathway is apparent. This is a major anti-apoptotic pathway that assures cell survival. Inhibitor of apoptosis protein 1 was also found to be induced, further emphasizing the importance of thrombin signaling for tumor cell survival. Interleukin 8 was the most strikingly upregulated gene in this experiment. The angiogenic properties of interleukin 8 indicates importance of thrombin signaling for the angiogenic switch in breast cancer.

**Conclusion:** Thrombin signaling in breast cancer cells influences anti-apoptotic and pro-angiogenic pathways.

#### 4. Characterize the profile of PAR-1 upregulated genes.

Hs578T express PAR-1 and PAR-2. Thrombin cleaves only PAR-1, but cleaved PAR-1 can crossactivate PAR-2<sup>5</sup> thus, the gene profile of thrombin stimulated cells may not properly reflect PAR-1 activation. Since PAR-1 has been associated with metastatic breast cancer, it was of interest to clearly define the genes that are selectively induced by PAR-1 stimulation. PARs can

be activated by synthetic peptides that correspond to their tethered ligand extracellular domain. The PAR-1 specific peptide TFLLRNPNDK was thus used to elicit PAR-1 specific responses in the subsequent experiments that utilized the larger 6800 gene chip from Affymetrix. Table 1 shows that thrombin responsive breast cancer cells also expressed TF, the cellular receptor that initiated the thrombin generating pathways. TF is known to support tumor cell metastasis<sup>6</sup>, but TF's role in breast cancer is incompletely understood. Under certain conditions, TF appears to be a tumor suppressor gene in breast cancer<sup>7,8</sup>. We recently found that the TF-VIIa-Xa coagulation initiation complex can activate PAR-1 directly and independent of thrombin generation<sup>9,10</sup>. It was therefore of interest to determine whether the responses to PAR-1 activation differ, if the cells are stimulated by a direct agonist or a protease that is presented by another cell surface receptor. Parallel ongoing work in the laboratory had also shown the power of gene chip technology in comparing the transcriptional response that results from stimulation of closely related receptors. The experiment was thus designed to compare the agonist responses to direct PAR-1 activation and to proteolytic cleavage of PAR-1 by the TF initiation complex. A 90 minute stimulation time was chosen for these experiments to capture both early (gene transcription related) and late (effector gene related) induction events. The experiment was repeated three times, and average gene induction was calculated for the repeats. Repeat experiments were found to be necessary to reduce the considerable noise associated with the fairly new array technology. Fig. 3 shows the comparison of gene induction by direct PAR-1 activation and by stimulation by TF-dependent proteolytic signaling through PAR-1.

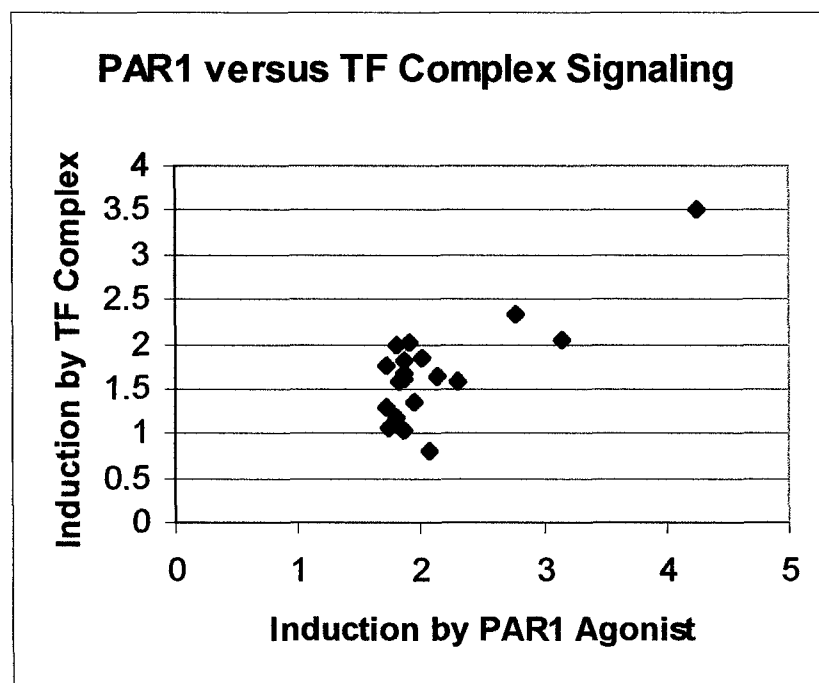


Fig. 3: Comparison of genes induced by direct PAR-1 activation and by PAR-1 activation by the TF complex in breast cancer cells.

Table 3 lists the most significantly induced genes on the human 6800 Affymetrix gene chip. The striking upregulation of interleukin 8 is apparent by both direct and TF-dependent PAR-1 activation, demonstrating that the previously observed thrombin response is due to PAR-1 activation. Endothelin 1 was detected as an additional transcript that was

highly induced by PAR-1 on this expanded gene set. Taken together with the upregulation of other genes that regulate endothelial cell function, these data strongly indicate that PAR-1 stimulation on breast cancer cells may influence angiogenesis. In addition, protective gene induction was also apparent. For example, A20 is a key gene known to counteract TNF $\alpha$

signaling that induces apoptosis. Another important finding from this experiment is the concordance in results when genes were represented by different probe sets on the array, as in the case of IL-8 and IL-6. This emphasizes the high reproducibility of the array technology in detecting changes in gene expression. It is notable that certain genes are not upregulated by TF-dependent protease signaling, but strongly induced by the PAR-1 agonist (e.g. ephrin B). We are following up on this observation with the hypothesis that a co-receptor, like TF, can modify PAR-1 responses and thus the consequence of thrombin signaling through PAR-1 in cancer biology.

Table 3: Gene induction by PAR-1 and TF complex dependent PAR-1 activation.

	Fold induction by	
	PAR-1 agonist	TF complex
Endothelin-1	4.2	3.5
IL-8	3.2	2.0
IL-8	2.8	2.3
28 ribosomal mRNA	2.3	1.6
FOSB	2.1	1.7
Collagen VI, alpha2	2.1	0.8
VALYL-tRNA SYNTHETASE	2.0	1.8
2		
c-myc-P64	1.9	1.3
Cox-2	1.9	2.0
Collagen VI, alpha2	1.9	1.0
EGR-3	1.9	1.6
DNA topoisomerase II binding protein	1.9	1.7
IL-6	1.9	1.8
NFKBIA	1.8	1.6
SERUM/GLUCOCORTICOID-REGULATED KINASE; SGK	1.8	1.2
IL-6	1.8	2.0
EPHRIN B2; EFNB2	1.7	1.1
endoglin	1.7	1.3
A20	1.7	1.8

## 5. Profiling for novel genes that are upregulated by PAR-1 signaling.

Stimulation with thrombin, the direct PAR-1 agonist and TF-complex dependent activation of PAR-1 resulted in the activation of similar known genes. We conclude from this observation that the experimental design was appropriate to capture relevant gene induction of the PAR-1 pathway. To characterize novel genes induced by PAR-1 stimulation, gene chips that are based on expression tagged (est) probes were used in subsequent experiments. Four separate arrays were available from Affymetrix to cover approximately 40,000 est clones. We reasoned that data mining of the est database was associated with a higher uncertainty than the chip design, based on well characterized genes, and anticipated a higher noise level in these experiments. We therefore chose to use all three experimental approaches, i.e. stimulation with thrombin, with the PAR-1 agonist, and with TF complex to profile for novel genes that are upregulated by PAR signaling in

breast cancer. We have completed the hybridizations and the quality control parameters demonstrate that valid data sets were obtained for all three conditions with at least two repeats each. Approximately 20 est probes showed highly reproducible upregulation per chip, indicating that up to 80 target genes were identified by the est search. The search for the gene products corresponding to these ests in the completed human genome sequences has yet to be completed.

### **Key Research Accomplishments**

1. Breast cancer cell lines were identified that showed highly reproducible PAR1 responses.
2. Gene profiling of the PAR1 response provides evidence for an involvement of PAR1 in regulating cell survival and angiogenesis.
3. Novel genes that are downstream of thrombin signaling in breast cancer were identified by large scale est screening.

### **Reportable Outcomes**

none

### **Conclusions**

1. PAR-1 expression was consistently observed in more malignant breast cancer cell lines.
2. PAR-1 induced genes are consistent with a cell survival response and PAR-1 antagonists may be considered as a potential avenue for treatment of metastatic disease.

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